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Laboratory tests and reference reagents employed in studies of inactivated hepatitis A vaccine

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Procedures to evaluate inactivated hepatitis A vaccines in volunteers have been examined. Solid-phase immunoassays were standardized with reference preparations and have been tested to measure antibody response to immunization and antigen content of vaccines. Following immunization, there was a good correlation between antibody response, determined with commercial immunoassays, and neutralization titres, as measured by the radioimmunofocus inhibition test. However, at lower titres of neutralizing antibody, the commercial immunoassay often yielded negative results. To improve the sensitivity of the immunoassay, the serum volume was increased. A fourfold increase of test serum resulted in greater sensitivity, increasing from 54 to 94%, while retaining 100% specificity. Further increases in the volume of test serum resulted in a loss of specificity. In a comparison of neutralization tests, similar titres of postvaccination sera were obtained by using the HM175 18f cytopathic strain of hepatitis A virus in a plaque reduction assay or the HM175 parental virus in the radioimmunofocus inhibition test. Use of the cytopathic virus obviates the need for radioactively labelled serum and reduces the time taken to conduct neutralization tests. The current laboratory procedures can meet the needs of large field trials of inactivated hepatitis A vaccines.

Keywords: Hepatitis A; immunoassay; neutralization test

INTRODUCTION

Solid phase immunoassays (SPIA) have made vital contributions to the control of hepatitis A. Initially, the antibody assays were used with a high degree of sensitivity and specificity for diagnostic and epidemiological purposes. This was due to the rapid, high and sustained antibody response following infection[1]. During studies on viral cultivation, antigen assays were employed to detect propagation and estimate virus concentration[2,3]. Immunoassays for antigen and antibody were extensively used during the development of inactivated vaccines and enabled rapid determination of viral antigen content and antibody response[4]. In contrast to natural infections, the antibody responses to the initial inactivated vaccines were modest and, at times, results of commercial immunoassays were negative although neutralizing antibody was present[5]. In addition, the radioimmunofocus inhibition test (RIFIT) is a highly labour-intensive neutralization test requiring 10–14 days to complete for comparatively small numbers of serum specimens. Therefore, attempts were made to increase the sensitivity of the immunoassays[6] and results are summarized in this report. To enable greater use of the neutralization test a cytopathic strain of hepatitis A virus (HAV) was also evaluated for use in determining the neutralizing antibody response to inactivated vaccines. In addition, this report briefly summarizes use of SPIA for measuring the antigenic content of inactivated vaccines.

MATERIALS AND METHODS

Antigen assay

HAV antigen content was determined by a solid phase radioimmunoassay (SPRIA) and by enzyme-linked immunoassay (EIA) as previously described[7,8]. A calibrated reference preparation was used to determine the antigen content[8].

Serum specimens

Pre- and postimmunization serum specimens were used from volunteers in previous institutionally approved protocols[5]. The volunteers had received two or more doses of inactivated HAV vaccine produced at

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Walter Reed or SmithKline Beecham Biologicals (Rixensart).

Immunoassay

The commercial RIA (HAVAB, Abbott Laboratories, Chicago, IL, USA) was used. Ten µl test serum and 200 µl competing labelled serum were used in the standard assay and modified tests were done with combinations of 20 and 190 µl, 40 and 170 µl, and 80 and 130 µl, of test serum and competing labelled serum respectively. A World Health Organization (WHO) reference hepatitis A immune serum globulin was used for control purposes[9].

Neutralization test

The RIFIT was used to titrate neutralizing antibody[10]. For a neutralization test with the cytopathic HM175/18f strain, virus was propagated in FRhK4 cells and the foci were detected by staining with crystal violet[11,12].

Statistical analysis

The regression approach to analysis of variance was used to test for parallelism of test groups[13].

RESULTS AND DISCUSSION

Vaccine antigen

Both SPRIA and EIA have been used to detect and measure HAV antigen content. The amount of antigen can be readily estimated by comparison with a standard curve (*Figure 1*)[6,8]. Slopes of dilution curves for crude harvest, purified and formalin-treated preparations were similar to that of the reference preparation. Formalin inactivation did not change reactivity. Results of statistical analysis for parallelism of the slopes of the four preparations supported this conclusion [f value of 2.54 with 3 and 9 degrees of freedom ($p = 0.12$)].

At present, each laboratory is employing its own reference preparation for which it has determined the antigen content. However, there is a need for a reference standard HAV antigen preparation for comparative and regulatory purposes. Studies for this purpose are being sponsored by the WHO. In addition, methods are needed to determine the HAV antigen content on alum-adsorbed vaccines.

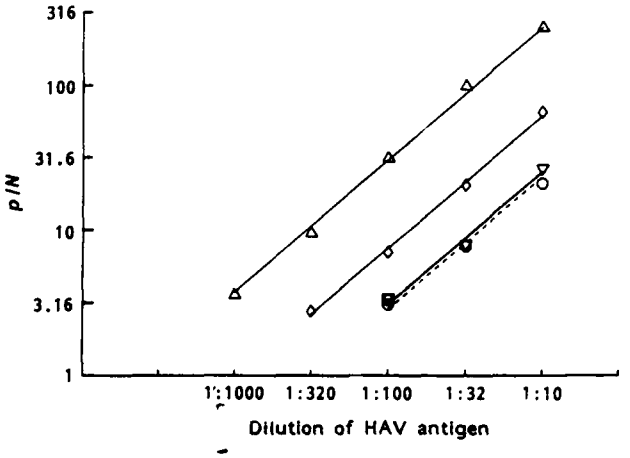

Figure 1 Radioimmunoassay for quantitative HAV antigen determination. ○, Standard; ▽, harvested HAV; △, purified HAV; ◇, formalin-inactivated HAV. *P/N*, Positive/negative

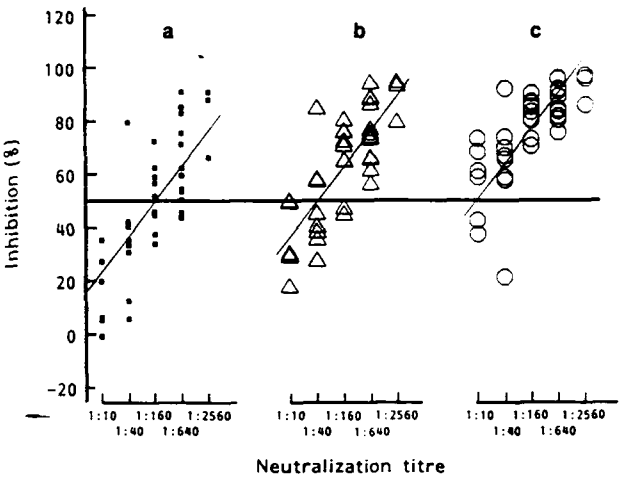

Figure 2 Relationship of the neutralization titres and standard and modified RIA for HAV antibody in vaccine recipients. Test volume: a, 10 µl; b, 20 µl; c, 40 µl

Table 1 Modified hepatitis A virus antibody RIA: percentage inhibition before and after inactivated vaccine in responding volunteers

Serum tested	Mean inhibition (%) and No. seropositive/total with test serum at			
	10 µl	20 µl	40 µl	80 µl
Pre-immunization	−12.5 (0/8)	−8.6 (0/8)	−5.7 (0/8)	23.1 (0/8)
Postimmunization	54.3 (5/8)	64.4 (7/8)	72.6 (8/8)	85.7 (8/8)

Neutralization titres ranged from 1:10 to 1:160, GMT = 1:40. With increasing volume of test serum the competing labelled serum was decreased to keep volume constant (210 µl)

Vaccine immunogenicity

Initial observations of antibody response to inactivated HAV vaccines indicated that there was a correlation ($r = 0.83$) between the neutralization titre and the percentage inhibition using standard commercial RIA which employs 10 µl of serum (*Figure 2*). However, many serum specimens with neutralization titres of ≤ 1:160 had RIA results below the minimum positive value of 50% inhibition. To improve the sensitivity of RIA, increased volumes of serum were tested from volunteers before and after immunization (*Table 1*). For this purpose, serum specimens with low titres of neutralizing antibody (1:10 to 1:160) were selected and the percentage inhibition was calculated by using 10 µl of the negative control serum supplied by the manufacturer. There was a progressive increase in percentage inhibition with greater volumes of test serum, which was in part due to the decreased amount of labelled competing serum. However, none of the eight pre-immunization serum specimens yielded positive results when the test volume was increased. After immunization, the number of seropositive specimens increased from five (with the standard test volume of 10 µl) to all eight (with 40 or 80 µl test serum).

To test specificity, standard and modified RIA was carried out with serum specimens from six vaccinated

Table 2 Modified hepatitis A antibody RIA: percentage inhibition before and after administration of inactivated vaccine in volunteers who did not develop neutralizing antibody

Serum tested	Mean inhibition (%) and No. seropositive/total with test serum at			
	10 µl	20 µl	40 µl	80 µl
Pre-immunization	9.4 (0/6)	19.9 (0/6)	23.4 (0/6)	32.1 (0/6)
Postimmunization	18.6 (0/6)	23.6 (0/6)	38.3 (0/6)	57.0 (5/6)[a]

Neutralization titre < 1:10
[a]2/6 seropositive when calculated from same volume of pre-immunization serum

Table 3 Modified hepatitis A virus antibody RIA: evauation in 43 volunteers given inactivated vaccine

Serum	Neutralization titre	No. seropositive/total and Inhibition (%)[a] with test serum at		
		10 µl	20 µl	40 µl
Pre-immunization	0	0/43 (1.1)	0/43 (0.8)	0/43 (12.3)
Postimmunization	0	0/5 (2.4)	0/5 (2.7)	0/5 (10.5)
	10	0/6 (15.5)	0/6 (34.2)	4/6 (56.9)
	40	1/8 (34.9)	3/8 (48.3)	7/8 (63.2)
	160	5/9 (51.9)	7/9 (66.3)	9/9 (81.7)
	640	10/12 (54.4)	12/12 (75.4)	12/12 (86.4)
	2560	3/3 (81.4)	3/3 (89.0)	3/3(92.6)
Neutralization titre for RIA positive		160	62	< 10

[a]Mean inhibition calculated with 10 µl negative reference serum

volunteers who did not develop detectable neutralizing antibody (*Table 2*). As observed above, none of the six pre-immunization serum specimens yielded positive results although the percentage inhibition increased by nearly 25% with the eightfold increased volume of test serum. However, five of the six postimmunization serum specimens had positive results, but only at the eightfold increased volume and when percentage inhibition was calculated by using the results with 10 µl reference serum. By using the value from 80 µl homologous pre-immunization serum to calculate the percentage inhibition, the number of positives was reduced from five to two. These findings suggested that modified RIA with 80 µl test serum would lose specificity but that volumes of ≤ 40 µl would achieve increased sensitivity without loss of specificity.

Further evaluation of modified RIA was carried out with serum specimens from 43 vaccinated volunteers who had neutralization titres ranging from < 1:10 to 1:2560 (*Table 3*). None of the 43 pre-immunization serum specimens had positive RIA results with any increased volume tested. There was a clear relation between the neutralization titre and the number of post-vaccination serum specimens that were RIA-positive. The mean 50% RIA conversion point was lowered from neutralization titres of 1:160 for the standard RIA using 10 µl to < 1:10 for modified RIA in which 40 µl serum was tested, thereby increasing the sensitivity of RIA by ≈ 16-fold. By using the same volume of homologous pre-immunization serum instead of the negative control serum to calculate the percentage inhibition, the number of seropositives was unchanged at each volume tested, with few exceptions. At both 10 and 20 µl test volumes, one additional positive was detected and, at 40 µl, there was one less. Analysis (*Figure 2*) of the data indicated that the slope of the relationship between neutralization titre and percentage inhibition was unchanged except that the y intercept had shifted to the left, indicating greater sensitivity. Statistical analysis of the data, using analysis of variance[13], supported the conclusion of a common slope ($f = 1.29$ and $p = > 0.05$).

The sensitivity of standard and modified RIAs to identify sera with HAV neutralizing antibody from volunteers given inactivated vaccine increased from 52% with 10 µl test serum to 94% using 40 µl, with 100% specificity (*Table 4*). However, the specificity fell to 17% with 80 µl test serum. Use of the homologous pre-serum value for 80 µl serum increased the specificity to 67%, a level that was still unacceptable. The findings indicate that modified RIA employing fourfold increased volumes of serum (i.e. 40 µl), achieved significantly increased sensitivity without loss of specificity and can be performed in a conventional test.

Recently, a cytopathic variant of HAV, HM175/18f, has been described which retained normal antigenicity and had a more rapid rate of replication[12]. Neutralizing antibody titres were similar with HM175/18f in plaque reduction neutralization tests (PRNT) and with parental HM175 virus using RIFIT (*Figure 3*). Of 46 postvaccination serum samples tested using RIFIT and PRNT titres of ≥ 1:10, 18(39%), had the same titre in both tests and an additional 23 (50%) had titres within fourfold. Thus, nearly 90% of the positive sera had titres that were

Table 4 Sensitivity and specificity of standard and modified RIAs for hepatitis A virus neutralizing antibody in volunteers given inactivated vaccine

Volume serum tested (µl)	Sensitivity No. positive/total (%)	Specificity No. negative/total (%)
10	26/50 (52)	11/11 (100)
20	36/50 (72)	11/11 (100)
40	47/50 (94)	11/11 (100)
80	12/12 (100)	1/6 (17)[a]

All pre-immunization sera were negative at each volume tested. Postimmunization serum with neutralizing antibody titre of ≥ 1:10 compared with 10 µl reference negative serum in RIA
[a]4/6 (67%) when calculated with 80 µl homologous pre-serum

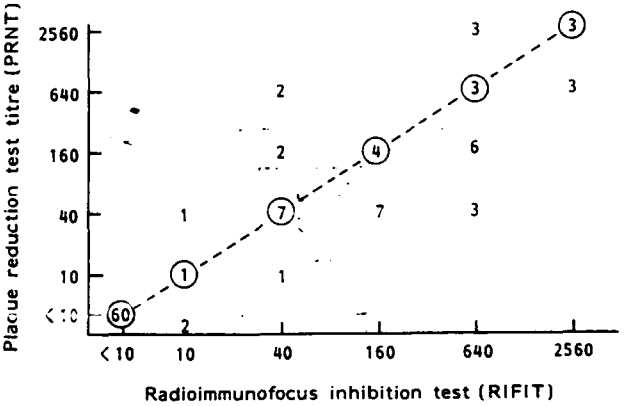

Figure 3 Comparison of the radioimmunofocus inhibition test and a plaque reduction test for neutralizing antibody in vaccine recipients using pre- and postimmunization sera. ○, No. of individuals with the same titre in both tests

within fourfold, reflecting a single test dilution; the remaining five (11%) had a 16-fold difference. The geometric mean titre (GMT) for RIFIT was 1:100 and for PRNT it was 1:72. All but one of the 53 pre-immunization sera were negative in both tests. The exceptional serum was reactive at a dilution of 1:10 using RIFIT. These results clearly indicate that PRNT with HM175/18f could replace RIFIT and eliminate the use of radioisotopes and thus achieve considerable savings in time and labour.

It will be important to evaluate the practice of using the WHO reference immune globulin as a standard preparation for quantifying antibody responses (expressed as mIU ml) in volunteers given inactivated vaccine. Errors may result from comparing a late immune response to infection with that to an inactivated purified vaccine at different times postimmunization. However, this widely available preparation does provide an initial reference value for estimating antibody response and enables comparison of different preparations and schedules. Detailed evaluation is required before critical comparisons can be made of the values obtained at different laboratories that use various procedures, i.e. RIA and EIA, to estimate the response in mIU/ml.

CONCLUSIONS

SPIA can be used to measure quantitatively the antigenic content of HAV preparations, from harvesting through purification and inactivation. Although standards prepared by each laboratory appear satisfactory for this purpose, there is a need for a reference standard to determine antigen content of vaccines made in different facilities. Standardized procedures should also be used to measure antigen content of vaccines containing alum.

There is a significant correlation between the neutralization titre and RIA values of serum specimens from volunteers given inactivated vaccines. RIA for detecting HAV antibody can be made more sensitive by a fourfold increase in the test volume of serum to 40 µl. This improves the sensitivity 16-fold without a loss of specificity. A modified RIA with 80 µl test serum appears to lose specificity.

PRNT employing a cytopathic strain of HAV (HM175/18f) gives similar titres to RIFIT, achieves considerable saving in time and labour and eliminates the use of radioisotopes.

Use of SPIA has enabled the rapid and quantitative evaluation of hepatitis A vaccines and will continue to play a vital role in vaccine production and use.

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